The Impact of Toll-Like Receptor 2 Genetic Variations on Susceptibility to Tuberculosis

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الخلاصه:
خلفية الدراسة: مرض التدرن هو مرض منتشر عالميا تسببه بكتريا *Mycobacterium tuberculosis* . وبالرغم من ذلك فان العديد من العوامل البيئية والوراثية يمكن أن تؤثر على حدوث وتطور المرض . ان لمستقبلات Toll-like 2 أهمية خاصة في الاستجابة المناعية لهذه البكتريا .
أهداف الدراسة : هدفت الدراسة الى استقصاء العلاقة بين تغايرين جينيين في جين TLR2 وهما Arg677Trp و Arg753Gln مع حدوث التدرن في المرضى العراقيين .
المواد وطرائق العمل : شملت الدراسة 55 مريض بالتدرن إضافة الى 30 شخصا سليما ظاهريا بنفس الأعمار كمجموعة سيطرة . جمعت عينات دم من مجتمع الدراسة واستخلص الحامض النووي . تمت مضاعفة جين TLR2 بطريقة تفاعل سلسلة البلمرة باستخدام بادئات خاصة . اجريت عملية التنميط الجيني بطريقة الكشف المباشر على تتابع القواعد .
النتائج : ظهر التغاير الجيني Arg753Gln بنمطين جينيين هما GG و AG في كل من مرضى التدرن ومجموعة السيطرة ، ففي مرضى التدرن بلغت نسبة هذين النمطين 85.45% و 14.55% على التوالي مقارنة مع 96.7% و 3.3% على التوالي في مجموعة السيطرة (نسبة الارجحية = 7.251 ، 95% فترة ثقة = 1.008 -59.211 ، P=0.035). أما التغاير الجيني Arg677Trp فظهر بنمط جيني واحد وهو CC .
الاستنتاجات : الأليل A للتغاير الجيني Arg753Gln يمكن ان يعد عامل خطورة للإصابة بالتدرن في المرضى العراقيين .

الكلمات المفتاحية : التدرن ، toll-like receptor2 ، التغاير الجيني ، *Mycobactrium*

Abstract
Background: Tuberculosis is a disease of worldwide distribution. This disease is known to caused by *Mycobacterium tuberculosis*, however several environmental and genetic factors can affect the occurrence and progression of the disease. Toll-like receptor 2 (TLR2) has a particular importance in immune response against this bacteria.
Aims: This study aimed to investigate the association of two single nuclotide polymorphisms(SNPs) in *TLR2* gene which are *Arg677Trp* and *Arg753Gln* with the incidence of TB in Iraqi patients.
Subject and Methods: A case-control study was conducted which involved 55 patients with confirmed pulmonary TB and other age-matched unrelated 30 healthy individuals as control group. Blood samples were obtained from each subject and DNA was extracted. The gene of *TLR2* was amplified with polymerase chain reaction (PCR) using specific sets of primers. Genotyping was achieved by direct sequencing.
Results: Only the SNP*Arg753Gln* appeared in two genotypes which were GG and AG in both TB patients and control groups. In TB patients, these genotypes account for 44 (85.45%) and 11 (14.55%) respectively, compared with 29 (96.7%) and 1 (3.3%) respectively in control group with significant difference (OR =7.251 95% CI=1.008-59.211, *P* = 0.035). The SNP *Arg677Trp* had only one genotype which was CC.
Conclusion: The allele A of the SNP *Arg753Gln* could be considered as a risk factor for TB among Iraqi patients.

Keywords: tuberculosis, *Mycobacterium*, toll-like receptor 2, polymorphism

Introduction

Tuberculosis is a cosmopolitan infectious disease infecting about one third of the world's population, although only 10% of those people develop clinical disease. One of the most important features of the causative agents (*M. tuberculosis*) is that they lack structurally variable strains, and have similar virulence capacity inside the host (Handzel, 2013). Accordingly, it is reasonable to assume that same morbidity and mortality occur in different populations when there are

similar environmental and socioeconomical conditions. However, there are wide variations in the prevalence of TB even in the world's regions with relatively similar conditions .This fact imposes genetic variations among populations and individuals that interfere with the outcome of the disease.(Brewer, 2000).

Immune system recognizes foreign bodies, including different pathogens, through what is called Pattern Recognition Receptors (PRRs). An important class of PPRs are TLRs which were found to play a crucial role in the induction of immune response (Netea *et al.*, 2012). In human there are 9 types of these receptors of which TLR2 has a special implication in the recognition of mycobacterial infection (Ryu *et al.*, 2006). Interestingly, two important polymorphisms in *TLR2* gene (*Arg677Trp* and *Arg753Gln)* have been extensively studied and were found to increase susceptibility to different infections such as *Borrelia* and cytomegalovirus (Tsehirren *et al.*, 2013; Joblondka *et al.*, 2014). The *Arg677Trp* polymorphism was found to be associated with lepromatous leprosy in Koran patients (Kang *et al.*, 2001) and pulmonary tuberculosis in Tunsian patients (Ben-Ali *et al.*, 2004), while the *Arg753Gln* polymorphism is associated with tuberculosis in Turkish patients (.,Ogus *et al* 2004). This study aimed to investigate the association of *Arg677Trp* and *Arg753Gln* polymorphisms in *TLR2* gene with the incidence of TB in Iraqi patients

Subjects and Methods

This retrospective case/control study included 55 patients with confirmed pulmonary tuberculosis (31 males and 24 females, age range 7-85 years, mean 69.6 ±9.76) who were attending Al-Hilla Consultant Clinic for Respiratory Disease/ Babylon Province/Iraq during the period from December 2013 to April 2014.

The specific criteria for enrollment were defined as the presence of at least one of the following: (1) clinical and radiological findings that indicate the presence of pulmonary TB, and at least one positive *M. tuberculosis* culture from three separate sputum examination, or one bronchial washing specimen obtained from bronchial scopy, (2) improvement in suspected pulmonary TB with empirical anti-TB therapy as indicated via clinical and radiological findings, (3) positive result for Xpert test which is a modern test for molecular detection of the causative bacteria in body fluid and (4) pathological evidence of TB as indicated from pleural or lung biopsy.

Family unrelated, apparently healthy 30 individuals from workers of the same hospital and from College of Medicine/ Babylon University were recruited to represent the control group. The mean age of control was 66.68±8.29 years. Exclusion criteria were defined as the presence of at least one of the following: (1) fever greater than 38.5 ^{o}C, (2) significant weight loss according to BMI calculation, (3) productive cough and night sweat for more than two weeks, (4) pregnancy or nursing an infant and (5) receiving an immuno-suppressive drug or cancer-related therapy.

Informed consents from patients as well as control were taken which included age, gender, smoking, body mass index (BMI), diabetes mellitus (DM), residence, and first relative family history TB.

Blood Samples and DNA Extraction

Three ml of venous blood was collected from each participant in EDTA tube which were kept at -20 ^{o}C until be used. DNA was extracted from these samples using ready kit (Favor prep DNA extraction mini kit/ Favor Gene Biotechnologies/ Taiwan) according to the manufacturer's instructions

PCR Protocols

Extracted DNA was used in PCR for amplification of two regions of *TLR2* gene. The primer set specific for *Arg677Trp* was F: GCCTACTG GGTGGAGAACCTT and R: CCAGTTCATACTTGCACCACT. The cycling conditions were an initial denaturation for 7 min at 95 ^{o}C, followed by 35 cycles of denaturation at 95 ^{o}C for 30 sec , annealing at 63 ^{o}C for 30 sec, extension at

72 ºC for 30 sec, followed by final extension at 72 ºC for 7 min with an expected fragment length of 199 bp. For PCR amplification of *TLR2 Arg753Gln* gene, the primer set was F: CCTGGCAAGTGGACCATTGAC and R: GGCCACTCCAGGTAGGTCTT. The cycling conditions were an initial denaturation for 5 min at 95 ºC, followed by 35 cycles of denaturation at 94 ºC for 30 sec, annealing at 55 ºC for 30 sec, extension at 72 ºC for 1 min, followed by final extension at 72 ºC for 7 min with an expected fragment length of 254 bp. A ready 50 µl PCR master mix (Bioneer/Korea) was used for preparing the PCR reaction. Template DNA (10 ng) from each sample and primers (5 ng from each) were added to each master mix tube. The mixture then put in shaker and spinner for 10 cycles for better mixing. Then, the mastermix tubes were transferred to the thermocycler (MyGenie 32 thermal block/Bioneer/Korea) which is previously programmed with the above protocols according to the gene to be amplified.

A 2% gel was prepared, and 10 µL aliquot of PCR product from each PCR tube was mixed with 2 µL loading dye and loaded into the wells of the gel. After 1 hour of electrophoresis, the gel was stained with ethidium bromide (Biobasic/Canada) (0.5 g/mL) for 20 min and examined using U. V. transilluminator with camera. The amplified products were determined by comparison with a commercial 1000 bp ladder (Kappa Biosystem/USA).

DNA Sequencing

Direct sequencing of PCR products was achieved in Bioneer company/Korea for DNA sequencing. The obtained sequences were aligned using clustalw software (available at www.genome.jp) with normal sequence from national centers for Biotechnology information (NCBI) and examined for presence of SNPs .

Statistical Analysis

The Statistical Package for the Social sciences version 14.0 (SPSS Inc., Chicago, USA) was used for statistical analysis. The polymorphisms were tested for deviation from Hardy-Weinberg Equilibrium (HWE) by comparing the observed and expected frequencies (Chi-square test). The association between genotype and risk factors with the incidence of TB was estimated by calculation of Odds ratio (OR) with 95% confidence interval (95%CI) using logistic regression. Statistical significance was set at a *P* value≤ 0.05.

Results

Risk factors

Table 1 shows the association of different risk factors with TB. Since the study intended to select control individual with age class that matches the TB patients, age appeared to have insignificant association with TB (OR=1.038, 95%CI=0.977- 1.104, p=0.229). Seven TB patients (12.73%) have first or second relative with TB compare to 1(3.3%) of control had these relatives. However, the association of family history with TB was insignificant (OR=2.229, 95%CI=0.495-36.14, p=2.009). Regarding gender, the disease seemed to have slightly higher prevalence among male (56.36%) than female (43.63%). However, the difference was insignificant (OR= 0.554, 95%CI=0.215-1.425, p=0.218).

The most prominent risk factor which appeared to have highly significant association with the prevalence of TB is the economic status. Taking the high economic status as a base for comparison, the prevalence of TB in both intermediate and low status differed significantly from that of control (OR= 35.0, 95%CI= 2.977-41.146 p= 0.005 and OR= 4.667, 95%CI= 1.241-17.549, p= 0.023 respectively).

Table (1): Association of risk factors with the incidence of TB

Risk Factors	Cases N=55	Control N=30	*P*-value	OR(95%CI)
Mean age in years (SD)	69.6 (9.76)	66.68 (8.29)	0.229	1.038 (0.977- 1.104)
Family history			2.009	
No	48 (87.27%)	29 (96.7%)		1.0
Yes	7 (12.73%)	1(3.3%)		2.229 (0.495-36.14)
Sex			0.218	
Male	31(56.36%)	21(70%)		1.0
Female	24(43.63%)	9(30%)		0.554(0.215-1.425)
Economic Status				
High	1(1.81%)	5(16.7%)	0.01	1.0
Intermediate	33(60%)	22(73.3%)	0.005	3.50 (2.977-41.146)
Low	21(38.18%)	3(10%)	0.023	4.667(1.241-17.549)
Mean BMI (SD)	21.44 (4.27)	24.71 (5.02)	0.047	1.78 (1.003- 1.098)
Smoking			0.035	
Never	36 (65.45%)	26 (86.7%)		1.0
Smoker (ex/current)	19 (34.54%)	4(13.3%)		3.431(1.043-11.281)
Diabetes Mellitus			0.09	
Non-diabetic	44 (80%)	28 (93.3%)		1.0
Diabetic	11 (20%)	2 (6.7)		3.5(0.721-16.982)
Residency			0.013	
Urban	16 (29.09%)	17 (56.7%)		1.0
Rural	39(70.91%)	13 (43.3%)		3.188 (1.261- 8.058)

BMI: body mass index, CI: confidence interval, N: number, OR: odds ratio, SD: standard deviation

Mean BMI among patients was 21.44 compared to 24.71 for control, and there was significant association with TB (OR= 1.78,95%CI=1.003- 1.098, p=0.047). Among TB patients, 19(34.54%) were found to be either ex-smoker or current smoker compared to only 13.3% in control group. Statistical test showed significant association between smoking and TB (OR=3.431, 95%CI=1.043-11.281, p= 0.035). Eleven TB patients (20%) had type 2 DM compared with only 2 (6.7%) among healthy control. However, the difference was not significance (OR=3.5, 95%CI=0.721-16.982, P=0.09). Residency is one of the most risk factor (other than polymorphism) which appears to have significant association with TB. Thirty one TB patients (70.91%) are living in rural areas compared to 13(43.3%) of control, whereas, only 16 (29.09%) of patients are living in urban areas compared to 17 (56.7%) of control (OR= 3.188, 95%CI=1.261-8.058, *p*= 0.013).

Detection of PCR Products

Gel electrophoresis of PCR product for *TLR2 Arg677Trp and Arg753Gln* genes are shown in figures (1) and (2) respectively.

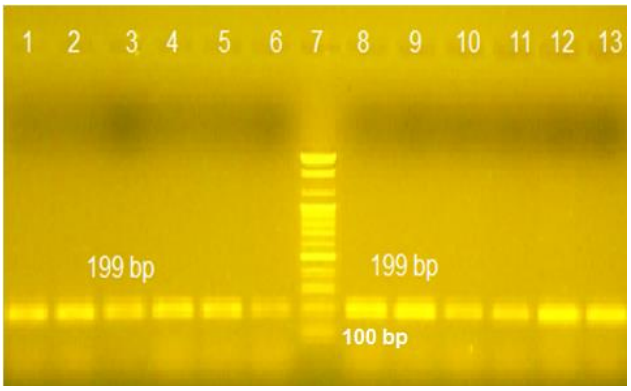


Figure (1): Agarose gel electrophoresis of PCR products from blood of control and TB patients for *TLR2Arg677Trp* with product size of 199. Lanes 1,2,3,4,5,6: positive result for *TLR2Arg677Trp* gene from blood of control, lane 7: DNA molecular size marker (100-2000 bp). Lanes 8,9,10,11,12,13: positive result for *TLR2Arg677Trp* gene from blood of TB patients

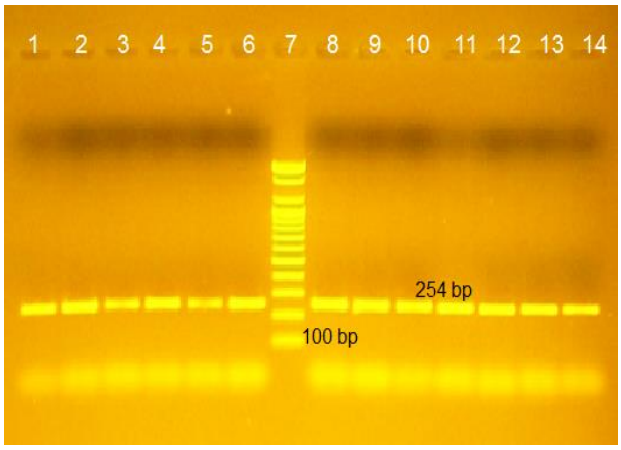


Figure (2): Agarose gel electrophoresisof PCR products from blood of control and TB patients for *TLR2Arg753Gln* with product size of 254 bp. Lanes 1,2,3,4,5,6: positive result for *TLR2Arg753Gln* gene from blood of control, lane 7: DNA molecular size marker (100-2000 bp), lanes 8,9,10,11,12,13,14: positive result for *TLR2Arg753Gln* gene from blood of TB patients

DNA Sequencing

Sequencing of the two genes was overlapped and the total sequencing involved part of *TLR2* spanning genetic location from Chromosome 4: 153704870 to Chr4: 153705165. According to NCBI, this stretch contains 23 SNPs. Of these SNPs, 16 are nonsynonymous while the remainders are synonymous. Fifteen of the nonsynonymous including *Arg677Trp* (Figure 3) appeared in single allele in both infected and control groups.

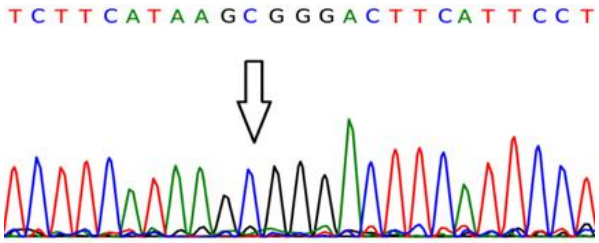
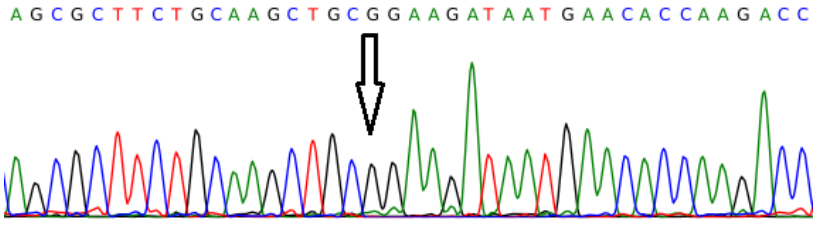
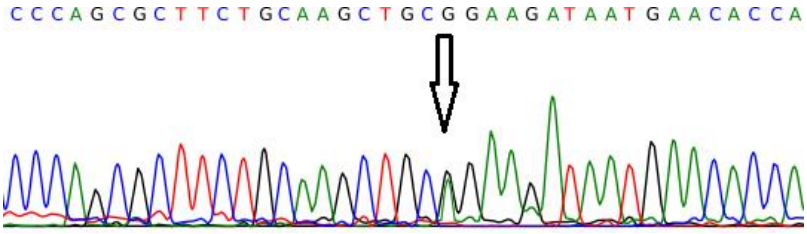


Figure 3: DNA sequencing for part of the third exon of *TLR2* rs121917864. The arrow indicates the position of the SNP Arg677Trp which appeared in only one genotype (CC).

The SNP rs5743708 (*Arg753Gln*) appeared in two genotypes: GG and AG in both TB patients and control groups (figure 4). In TB patients these genotypes account for 44 (85.45%) and 11 (14.55%) respectively, compared with 29 (96.7%) and 1 (3.3%) respectively in control group with significant difference (OR =7.251 95% CI=1.008-59.211, *P* = 0.035).



(A)



(B)

Figure (4): DNA sequencing for part of the third exon of *TLR2* rs5743708. The arrow indicates the position of the SNP Arg753Gln.A: represents the genotype GG; B: AG

Using chi-square for testing allele distribution, the result indicated that the SNP met Hardy-Weinberg equilibrium in both patients and control. Allele's analysis confirmed the aforementioned results (table 2). The frequencies of G allele (wild) among patients and control groups were 90% and 98.3% respectively, while the frequency of A allele (mutant) among patients and control were 10% and 1.7% respectively with significant difference (*P*= 0.043).

Table (2): Genotypes and alleles of (SNP ***rs5743708***)

Variables	Cases N=55	Control N=30	*P*-value	OR(95%CI)
rs5743708				
GG	44 (85.45%)	29 (96.7%)	0.035	1.0
AG	11 (14.55%)	1 (3.3%)		7.25 (1.008-59.211)
Allele				
G	99(90%)	59(98.3%)	0.043	1.0
A	11(10%)	1(1.7%)		6.556(1.002-52.073)

N: number, OR: odds ratio, CI: confidence interval

Discussion

The study revealed highly significant association between the heterozygous (AG) genotype of the SNP Arg753Gln with the susceptibility to TB. That is implies that carriers of this genotype have 7.251-fold risk of getting TB compared with homozygous geneotype carriers under the same circumstances. This significance was further confirmed by allele analyzing which indicated that mutant allele (A) was significantly associated with TB (*P*=0.043). This result is in accordance with many previous works. In neighboring countries, Dalgic *et al*. (2011) in Iran, found an association between Arg753Gln polymorphism and TB. More recently Ferhad et al. (2014) found that allele G (wild type allele) of this SNP decreased significantly in TB patients compared with the control group. In Turkey, Ogus et al. (2004) found 4.7% frequency of A allele of Arg753Gln (1.7% homologous and 6% heterologus) among Turkish population (6 and 1.6-fold for carriers of AA and GA genotype respectively). Among Arab countries, Ben-Ali et al. (2004) have demonstrated an association between the SNP *Arg677Trp* but not *Arg753Gln* with TB in Tunisian population. However, Ajili *et al*. (2010) did not detect any of the two SNPs among the same population. Globally, Xue *et al*. (2009) in South China and Selavaraj *et al*. (2010) in South India did not find such association. It seems that variation in ethnic and geographic origin of each population determine the prevalence of this and other SNPs among the population and subsequently the association with certain diseases (Loana *et al*., 2012).

TLR2 is encoded by a DNA sequence composed of 2352 bases that specify 784 amino acids (Rook and Hernandez-Pando., 1996). The characteristic feature of this receptor is the presence of an extracellular leucine-rich domain (amino acids 1-588), a single transmembrane domain (amino acids 589-609), and a cytoplasmic domain (amino acids 610-784) (Texereau *et al*., 2005).

Most previous studies focused on the association of TLR2 polymorphism in two certain SNPs (Arg677Trp and Arg753Gln) with the incidence of various diseases including TB. That is because certain allele of these SNPs reduce the activation of nuclear factor kappa light chain of B cells (NFκB) and then increased the risk of infection (Texereau et al., 2005). In fact, the current study does investigate the association of these two SNPs with the incidence of TB. However, the results indicated the presence of only one of them (Arg753Gln) and the absence of the other.

There are many hypothetical mechanisms by which this polymorphism can increase the susceptibility to infection with *M. tuberculosis* and may be other infectious agents. First of all, it is nonsense to suppose that this SNP can affect the recognition efficiency of TLR2 because the SNP is located within intracytoplasmic domain of the receptor, while the sensation and recognition of PRPs is the function of extracellular

domain of the receptor. One of the suggested mechanism is that mutant allele could diminish the expression of TLR2. However, this SNP is located in the third exon away from the gene promoter region and therefore it is unlikely to affect the transcription of the gene. Furthermore, this assumption was practically confuted by Xiong *et al* (2012) who transfected human embryonic kidney (HEK293) cell line with Arg753Gln and wild type, and used real-time PCR for quantitative detection of mRNA of mutant and wild genes. They found no differences in the expression of these genes.

Another mechanism which can be accused to influence the normal function of TLR2 is disruption in tyrosine phosphorylation. TLR2 leads to NFκB-mediated transcription through two pathways: NFκB translocation and NFκB transactivation (Finberg *et al.*, 2012). While the first pathway does not involve tyrosine phosphorylation, the second pathway employs at least one tyrosine phosphorylation event for active signaling. This pathway involves the association of TLR2 with p85 subunit of phosphotidylinositol-3 kinase (PI3K), Myeloid differentiation protein (MyD88), Rac1 and B cell tyrosine kinase (Btk) (Liljeroos *et al.*, 2007). Two highly conserved tyrosine residues in the intracellular domain of TLR2: Y616 and Y761 have been implicated in the phosphrylation of the receptor (Arbibe *et al*., 2000). The substitution of positively charged arginine with neutral glutamine may create an alteration in the conformation and electrostatic potential of the TLR2 domain, and subsequently the recruitment of protein tyrosine kineses or the accessibility of tyrosine residues to tyrosine kinases (Finberg *et al*., 2012).

As TLR2 singling employs two pathways, the disruption in tyrosine phosphorylation does not seem to fully explain the functional impairment of these receptors, and there must be another activity of TLR2 which may be affected by *Arg753Gln*. The candidate activity is the heterodimerization with TLR6 since this heterodimerization is the initial step for cell activation in response to *M. tuberculosis* (Bowdish *et al*., 2009;., Drage *et al* 2009). The molecular TLR2-TLR6 dimerization is not fully understood. However, one hypothesis of heterodimerization postulates that the dimerization interfece involves the interaction of DD loop region of TLR2 and the BB loops of TLR6 or TLR1 (Basith *et al*., 2011). Regardless of the mechanism by which the dimerization occurs, the result of this dimerization is formation of a scaffold to which TIRAP/Mal and MyD88 adapters and different kinases, including protein tyrosine kinases and IRAKs, are recruited (Chockalingam *et al*., 2012).

Since the *Arg753Gln* is localized within TIR domain, it could interpret with dimerization may be through imposing changes in electrostatic potential with DD loop which affects the residues involved in TLR2 interaction with TLR6. Using western blot analysis, Xiong *et al*. (2012) found significantly lower ability of the *Arg753Gln* in association with TLR6, a fact which support the later hypothesis.

While the exact mechanism by which *Arg753Gln* could affect the normal function of TLR2 is a matter of debate, the result of the current study suggests that this polymorphism could be considered as a risk factor for TB. However, further studies with larger sample size are needed to obtain solid conclusion.

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